

## Association of BoLA-DRB3.2 alleles with tick (*Boophilus microplus*) resistance in cattle

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**ABSTRACT.** Losses caused by bovine tick burdens in tropical countries have a tremendous economic impact on production systems. Besides reducing production, this parasite can cause death in the most susceptible animals. The use of commercial acaricides has been the major method of control, but their misuse has led to tick resistance to many chemicals. More recently, vaccines have been used in some countries without solving the problem completely. An alternative could be the development of resistant animals and the use of genetic markers and candidate genes that could help with the enormous task of selecting resistant animals. The bovine lymphocyte antigen genes (BoLA) have been shown to be associated with some parasitic infestations and disease incidence. Thus, the objective of the present study was to determine the association of BoLA-DRB3.2 alleles with tick resistance in cattle. The study was conducted on 231 F2 (Gyr x Holstein) animals that were artificially infested with 10,000 tick larvae. Log of tick count +1 was used as the dependent variable in a mixed animal model with allele sub-

stitution effects in addition to fixed effects of year and season at tick count, sex of calves, age of animal at tick count, hair type (short-straight, short-curl, long-straight, and long-curl), coat color (white, >75% white, 50- 75% white, and 25-50% white), and additive genetic, permanent environmental and residual effects as random. Females showed fewer ticks than males. Animals with short-straight hair were more resistant to tick infestation than animals with long-curl hair, and animals with whiter coat color also had fewer ticks. An association between BoLA alleles and lower tick number was found for alleles DRB3.2 \*18, \*20 and \*27 at the 5% significance level. Also, one allele (DRB3.2\*16) showed an association at the 10% level. Allele \*27 was the most frequent in the population (30.7%), followed by alleles \*16 (10.8%), \*20 (8.7%) and \*18 (2.4%). These results suggest that BoLA-DRB3.2 alleles could be used to help in the selection of animals resistant to tick infestation. However, further studies involving a larger population of cattle in combination with other BoLA genes may help to understand the mechanisms of resistance to parasites.

**Key words:** BoLA, Tick resistance, Bovine, Cattle, Candidate genes, Molecular markers

## INTRODUCTION

Around a billion cattle, mainly located in tropical regions, could be afflicted by various tick species or by the diseases they transmit, which could lead to significant loss in production systems (Pegram et al., 1991). In these regions, infection by these parasites, besides reducing production, could cause the death of the more susceptible animals. In most Latin American countries the predominant species of bovine ticks is *Boophilus microplus*.

In Brazil, which has the largest commercial bovine population in the world - around 170 million animals - losses caused by ticks and tick-related diseases are estimated to total US\$800 million/year. Honer and Gomes (1990) estimated that cattle infected with ticks and worms could lose from 18 to 47 kg of live weight per year. In Australia, Frisch et al. (2000) estimated that an animal with an average of 40 ticks/day could lose weight equivalent to 20 kg/year. Also, they reported that animals infested with more than 200 ticks for a period of six weeks could die if not treated.

The effect of tick infestation on milk production is also reported in the literature. In Brazil, Furlong et al. (1996) estimated a reduction of 23% in milk yield/day when crossbred Holstein-Zebu cows were infested with an average of 105 ticks. Also, Teodoro et al. (1998) reported a reduction of 529 kg (26%) of milk/lactation in Holstein cows untreated with acaricides. In Australia, Holstein cows of high production were submitted to an increasing burden of ticks every week for a total period of 105 days. In the last week, these cows showed a reduction of 2.86 kg of milk/day and 10.6 kg of live weight (Jonsson et al., 1998).

For many years, tick control has been mostly based on the use of acaricides; however, their misuse has given rise to ticks resistant to pesticides and also increased environmental contamination. Vaccines have been used in some countries without solving the problem completely (Labarta et al., 1996; Frisch, 1999). Another alternative could be the use of resistant animals. However, the identification of resistant animals is costly and time consuming. The identification of molecular markers associated with tick resistance in cattle could be used in marker-assisted selection.

The bovine lymphocyte antigen complex (BoLA) has been studied extensively for the past 20 years because of its importance in host immunity (Spooner et al., 1978). The Class I and Class II BoLA genes encode proteins mediating antigen presentation (Anderson, 1990; Teale et al., 1991). Studies have demonstrated associations between BoLA alleles and disease incidence. The Class I and Class II BoLA genes have been associated with the incidence of mastitis (Mejdell et al., 1994; Dietz et al., 1997; Kelm et al., 1997; Sharif et al., 1998), bovine leukemia virus infection (Roth and Kaeberle, 1981; Xu et al., 1993; Skow et al., 1998), chronic posterior spinal paresis (Park et al., 1993) and parasitic load (Stear et al., 1988).

Investigations relating BoLA genes and tick resistance are rare. Stear et al. (1990) studied the relationship between ectoparasites (*B. microplus* ticks) and BoLA Class I antigens and showed that animals with the BoLA antigens W6.1 and W7 had significantly fewer ticks than animals lacking these antigens. Martinez et al. (2004) in a preliminary study, found a putative association between tick count and alleles \*10 and \*42 ( $P < 0.1$ ) of the BoLA-DRB3.2 gene. They also reported a relationship between warble count and alleles \*31 ( $P < 0.05$ ), \*42 ( $P < 0.10$ ) and \*51 ( $P < 0.05$ ) in the same population.

The objective of the present study was to determine the association of BoLA-DRB3.2 alleles with tick resistance in an F2 crossbred Holstein-Gyr population.

## MATERIAL AND METHODS

### Animals

A total of 344 infestations in 231 F2 animals were studied to assess tick resistance. The F2 population was produced by crossing F1 females (50% Gyr: 50% Holstein) with F1 sires of the same genetic composition. The F1 cattle were generated by embryo transfer in 78 Gyr cows mated with four Holstein sires resulting in a total of 150 F1 animals (males and females). Of those, only 4 F1 sires were chosen according to their performance to mate with 68 F1 females, avoiding relationships among sires and cows.

All F2 animals were raised together on an experimental farm located in Valença, State of Rio de Janeiro, Brazil, in a hilly region at an altitude between 200 and 400 m above sea level. The climate corresponds to Cwa of Koppen's classification (Koppen and Geiger, 1936) (mild, dry winter, hot summer), with the dry season extending from April to September (Teodoro and Madalena, 2003). The experimental calves were artificially reared on approximately 4 L of whole milk/day and housed individually up to 8 weeks of age in an area free of ticks. From this age up to 6 months, they were kept in paddocks of *Cynodon dactylon* L, being fed up to 2 kg/head/day of 18% crude protein ration plus chopped elephant grass (*Penisetum purpureum*, Schumacher). In these paddocks they started to have contact with ticks.

Thereafter, they were allowed to graze on pastures of predominantly *Brachiaria decumbens* (Stapf.) supplemented with chopped elephant grass plus 1 kg/head/day of concentrate in the dry season and periods of pasture shortage. Minerals were made available in the paddocks for all animals. During the period from birth to 10 to 14 months of age the F2 animals were not treated with any product to control ticks.

Animals were studied in contemporary groups with age ranging from 10 to 14 months. To determine tick resistance, each F2 animal was artificially infested with approximately 10,000 tick larvae by placing them in the “dorsal-lumbar” region of the animals. Animals were kept tied up for 30 min to prevent grooming and to allow the larvae to spread to all regions of the body. Afterward, they were kept on pastures for 21 days when the tick count was conducted. All engorged female ticks 4.5 to 8.0 mm in length were counted on only one side of the animal, and the count was then multiplied by two. Infestations were carried out during the spring, summer and fall seasons, allowing some animals to be studied in two different seasons.

### DNA genotyping

Blood samples were taken from all F2 animals to extract DNA to genotype for the BoLA-DRB3.2 alleles. DNA samples were obtained using a phenol-chloroform and proteinase K procedure. DNA quality and concentration were determined by UV spectroscopy.

Amplification of the BoLA-DRB3.2 alleles was accomplished by a nested PCR procedure as described by van Eijk et al. (1992). The primers HLO30 (5'-ATCCTCTCTGCAGCACATTTCC-3') and HLO31 (5'-TTAAATTCGCGCTCACCTCGCCGCT-3') were used for the first amplification. The primers HLO30 and HLO32 (5'-TCGCCGCTGCCACAGT-3') were used in the second amplification to increase PCR specificity and to decrease the presence of heteroduplexes. The HLO32 primer consists entirely of nucleotides from the 3' end of exon 2 and has 8 nucleotides overlapping with primer the 3' end of HLO31.

The first amplification reaction consisted of 10 cycles of 94°C (60 s), 60°C (120 s) and 72°C (60 s). The PCR mixture contained 12.5 ng of template DNA, one unit of *Taq* polymerase, 100 µM of dNTPs, 0.2 µM of each primer, 10 mM Tris-HCl, pH 8.0, 2.5 mM MgCl<sub>2</sub>, and 50 mM KCl. The second amplification reaction mixture consisted of 30 cycles of 94°C (60 s), 65°C (30 s), using 1.4 µL from the first amplification reaction as DNA template in a final volume of 35 µL with one unit of *Taq* polymerase, 100 µM of dNTPs, 0.2 µM of each primer, 10 mM Tris-HCl, pH 8.0, 2.5 mM MgCl<sub>2</sub>, and 50 mM KCl. PCRs were carried out using the GeneAmp PCR System 9600 (Applied Biosystems).

The PCR products generated in the second amplification reaction were visualized by loading 5 µL onto native polyacrylamide gels (5%, 500 volts, 1 h) to standardize DNA quantity as well as to detect amplification problems. Afterward, 10 µL of the second amplification reaction was digested for 2 h at 37°C with 5 units of *RsaI* and *HaeIII* restriction enzymes and for 2 h at 60°C with the enzyme *BstI* in a final volume of 15 µL. Digested fragments were loaded onto native polyacrylamide gels (12%, 800 volts, 3 h) using 35-cm tall plates to assure perfect separation of the alleles. Gels were silver-stained and fragments were scored using 10- and 25-bp ladders. Fragment nomenclature followed van Eijk et al. (1992), BoLA Workshop Nomenclature Web Site (<http://www.projects.roslin.ac.uk/bola/wk92b.html>) and Maillard et al. (1999).

### Statistical analysis

Allele frequencies of the BoLA-DRB3.2 gene for the F2 animals were calculated by direct count. Analysis of the data was performed by the following animal mixed model:

$$Y = Xh + Za + Zp + Mm + E$$

where: Y is the vector of log count +1; X and Z are matrix of zeros and ones related to the fixed and random effects; h, a, p, and E are the solution vectors to the fixed, additive genetic, permanent environmental, and residual effects, respectively; m is a vector that includes the regression effects of allele substitution of the BoLA gene, and M is a matrix of 0, 1 or 2 showing the number of copies present in each animal. Fixed effect includes year and season at counting, age of animals at counting, sex, hair type, and coat color. Additive genetic, permanent environment and residual effects were assumed to be randomly distributed with zero means and variances  $\sigma_a^2$ ,  $\sigma_p^2$  and  $\sigma_e^2$ , respectively. A complete relationship matrix with parents and grand-parents of the F2 animals were used. Analysis was carried out using the PROC MIXED of the SAS Institute (2002) package. Data distribution and variable description are given in Table 1.

**Table 1.** Number of animals, number of evaluations, number of ticks, and their minimum and maximum by class of fixed effects.

Variable	No. of animals	No. of evaluations	Number of ticks		
			Average <sup>1</sup>	Minimum	Maximum
<b>Year</b>					
2000	53	77	27.2	0	128
2001	71	117	40.1	0	290
2002	74	117	44.7	2	274
2003	33	33	18.6	2	64
<b>Season</b>					
October-March	99	165	35.2	0	274
April-September	132	179	38.5	0	290
<b>Sex</b>					
Female	120	176	32.4	0	214
Male	111	168	41.6	0	290
<b>Hair type</b>					
Short-straight	114	173	27.7	0	214
Short-curl	47	68	44.2	0	214
Long-straight	52	81	46.1	0	280
Long-curl	18	22	52.8	10	172
<b>Coat color</b>					
Totally white	22	30	18.7	0	68
More than 75% white	52	74	28.6	0	188
From 50 to 75% white	75	110	45.4	0	290
From 25 to 50% white	82	130	38.7	0	246

<sup>1</sup>Ordinary means estimated by PROC MEANS of SAS Institute (2000) package.

## RESULTS

Twenty BoLA-DRB3.2 alleles were found in the F2 population used in this study (Table 2). The most frequent one was allele \*27 (30.74%), followed by alleles \*16 (10.82%), \*3 (9.31%), \*23 (9.09%), and \*20 (8.66%). Some of them showed very low frequencies (alleles \*6, \*12, \*35, \*42, \*44, \*47, and \*51) and were eliminated from association studies. In Table 3, the analysis of variance (ANOVA) showed high level of significance ( $P < 0.01$ ) for all effects except for the season at counting. The non-significant effect of season may be due to the fact that animals were artificially infested, and therefore, the level of tick infestation was independent of climate conditions during the year. Also, three alleles (\*18, \*20 and \*27) showed significant correlation with decreased number of ticks per animal at the 5% level, and allele \*16 showed significance at the 10% level.

**Table 2.** BoLA-DRB3.2 allele frequencies in a Gyr x Holstein F2 population.

Alleles	Frequency (%)
*3	9.31
*5	7.79
*6	0.87
*10	1.52
*11	5.19
*12	0.22
*15	2.81
*16	10.82
*18	2.38
*20	8.66
*22	4.11
*23	9.09
*27	30.74
*31	1.73
*34	1.73
*35	0.65
*42	0.87
*44	0.43
*47	0.22
*51	0.87

The least square means for the log count +1 (Table 4) indicated that males had more ticks than females. Some authors have observed the same sex effect and have suggested that it could be due to hormone differences (Stear et al., 1990; Veríssimo et al., 1997). Similarly, hair type and coat color had a significant effect in the log count of ticks per animal. There was a great variation in these effects, from 2.58 to 3.62 and from 2.66 to 3.29 for hair type and coat color, respectively. Animals with short-straight hair type showed fewer ticks than all the others. A possible explanation is that ticks may have more difficulty in attaching to this type of hair and that it is easier for the animal to groom itself (Veríssimo et al., 1996). Also, animals with a whiter coat had fewer ticks than darker coat animals. Since ticks are dark-colored, one explanation is

**Table 3.** ANOVA mean squares and level of significance for the log count +1.

Source of variation	Degrees of freedom	Mean squares
Year	3	9.24**
Season	1	0.32
Sex	1	6.83**
Hair type	3	7.49**
Coat color	3	4.85**
Age at count	1	7.25**
Allele <sup>1</sup>		
*3	1	0.08
*5	1	0.01
*10	1	1.44
*11	1	0.89
*15	1	0.42
*16	1	2.86 <sup>+</sup>
*18	1	3.64*
*20	1	4.57*
*22	1	0.05
*23	1	0.27
*27	1	3.43*
*31	1	0.37
*34	1	0.02
Error	319	0.92

<sup>1</sup>Only alleles with frequency >1% were analyzed.

\*P < 0.05, \*\*P < 0.01, <sup>+</sup>P < 0.10

that in darker animals, they would be protected against predators such as birds. To our knowledge similar results have not been previously published. We investigated the possibility of an interaction between hair type and coat color that could explain this effect, but the results were not significant.

The association between BoLA alleles and total tick count measured as the log count +1 regression coefficient is presented in Table 5. The results indicated a significant ( $P < 0.05$ ) decrease in the total number of ticks when alleles \*18, \*20 or \*27 were present. Also, in the presence of allele \*16, there was a near-significant trend ( $P < 0.10$ ) toward a decreased number of ticks/animal. Allele \*18 had the greatest effect (-0.57) in decreasing the log count of ticks/animal. Although, allele \*27 had a smaller effect (-0.28) compared with all the others, it is important to note that it was the most frequent in the population (30.74%) (Table 2). Selection of animals with allele \*27 could lead to a rapid response, resulting in a decreasing number of ticks per animal as their progenies become increasingly resistant. Similarly, selecting parents possessing alleles \*18, \*20 or \*27 for future generations should increase the stock's resistance to tick infestation.

## DISCUSSION

Although selection for moderately to highly heritable traits such as production and carcass traits has been successful in cattle, attempts to decrease health problems using genetic

**Table 4.** Least square means and standard error for log count +1 for year and season at count, sex, hair type, and coat color.

Variables	Least square means	Standard error
Year		
2000	3.05	0.14
2001	3.26	0.11
2002	3.54	0.12
2003	2.33	0.20
Season		
October-March	3.01	0.11
April-September	3.08	0.09
Sex		
Female	2.89	0.10
Male	3.19	0.10
Hair type		
Short-straight	2.58	0.09
Short-curl	2.97	0.14
Long-straight	3.01	0.12
Long-curl	3.62	0.22
Coat color		
Totally white	2.66	0.19
More than 75% white	2.92	0.12
From 50 to 75% white	3.31	0.11

**Table 5.** Log count +1 regression coefficients<sup>1</sup> for associations between BoLA-DRB3.2 alleles and total tick count.

Allele <sup>2</sup>	B	Standard error
*3	-0.05	0.16
*5	-0.01	0.19
*10	+0.45	0.36
*11	+0.22	0.22
*15	+0.17	0.25
*16	-0.30**	0.17
*18	-0.57*	0.29
*20	-0.34*	0.15
*22	+0.05	0.21
*23	+0.121	0.22
*27	-0.28*	0.14
*31	-0.20	0.31
*34	-0.05	0.35

<sup>1</sup>A positive sign indicates that the allele was associated with an increase, and a negative sign indicates that the allele was associated with a decrease in the total number of ticks per animals.

<sup>2</sup>Only alleles with frequency >1% were analyzed. \*P < 0.05. \*\*P < 0.10.

selection have only achieved moderate success (Lie and Solbu, 1981; Stear et al., 1988; Frisch et al., 2000). In dairy cattle, selection for improved health has been hampered by low heritabilities among both direct and indirect health measures (Emanuelson et al., 1988). In poultry, selection based on MHC type has been successful in reducing the incidence of some specific diseases, and the potential for MHC-assisted selection has been demonstrated in a variety of other species (Warner et al., 1987). Such a marker system for disease susceptibility and parasite resistance could greatly facilitate selection for improved general health in cattle. In addition, marker information could be useful when selecting among animals of equivalent ancestor merit, such as selection among full-sib bulls in a MOET program. The results obtained in this study, along with those from other authors (Stear et al., 1990; Weigel et al., 1990; Dietz et al., 1997; Martinez et al., 2004), confirm the potential of BoLA alleles as molecular markers for health and production traits in cattle. However, particular allelic effects should not yet be considered conclusive.

Another major study evaluating the association between tick resistance and BoLA alleles was conducted in Australia (Stear et al., 1984, 1989, 1990). It was reported that calves with BoLA antigen W6.1 or W7 clearly had fewer mature *B. microplus* larvae compared to calves lacking these antigens. Our results also indicate a reduction in tick number/animal when the BoLA-DRB3.2 alleles \*18, \*20, or \*27 were present. Although our results with Class II BoLA cannot be directly compared with the Australian Class I BoLA results, they clearly show that the BoLA complex plays some role in the animals' general health system making some animals more resistant to tick infestation than others.

Some theoretical considerations suggest that Class II gene products may be more important in the expression of resistance than Class I gene products. Immediate hypersensitivity reactions at the site of tick attachment stimulate the removal of ticks by grooming. Animals prevented from grooming show a transient increase in tick numbers (Willadsen, 1980). Studies in humans suggest that Class II genes are more important than Class I genes in the regulation of the immediate hypersensitivity reaction (Freidhoft et al., 1988). Several authors (Kemp et al., 1971; Allen, 1973; Bagnall and Double, 1975) attribute the rejection of ticks by their hosts to the combined action of basophils, eosinophils, T lymphocytes, and IgG since such cells and particularly basophils predominate in the inflammatory reaction. However, basophils are recruited from the bone marrow, via the blood stream, to be activated locally at the point of the tick's attachment to the host's skin. These cells require a time interval of about 6 h, which is the minimum period elapsed before they are detected in the inflammatory infiltrate (Moraes et al., 1992). On the other hand, mast cells reside in the dermis and their degranulation can be triggered by the combined mechanical trauma caused by the tick's mouth pieces and its injected saliva. In Zebuine hosts, undamaged skin has been found to harbor more than twice as many mast cells per surface unit than in the Taurine's uninjured integument (Moraes et al., 1992). The degranulation of these cells liberates several pharmacologically active substances such as histamine among others which cause intense pruritus. This teases the animals into self-licking, becoming the main mechanism by which they get rid of most tick larvae. Moraes et al. (1992) reported that the self-cleaning activity through self-licking starts at half an hour to two hours after tick infestation. Therefore, this reaction begins long before basophils appear in the blood stream (about 6 h) after the tick stimulus elicited by the parasite's initial bites. The evidence found by Moraes et al. (1992) of a much higher number of mast cells in the uninjured skin of Zebuines as compared to the number of these cells in the uninjured skin of taurine animals may explain - at least in part - the higher efficiency at which Zebuines get rid of their ticks when compared to Taurine animals.

The immediate hypersensitivity reaction is mediated by IgG and IgE, and is associated with an increase in vascular permeability and corresponding edema due to local mast cell degranulation. In fact, mast cells can be seen in significant numbers at *Rhipicephalus sanguineus* feeding sites on dogs (Szabó and Bechara, 1999). These mast cells are probably coated with IgE or IgG following the first exposure to ticks, and may degranulate upon challenge with tick antigens.

Although there are no results directly associating the BoLA complex with the number of mast cells, there is some indication of an association between the BoLA-DRB3.2 alleles \*11, \*24, \*12, \*3, and \*28 and increased serum IgG2 concentration, and between allele \*26 and decreased serum IgG2 (Dietz et al., 1997).

Further studies involving a larger population of cattle in combination with other markers in the BoLA region may help to strengthen the interaction between the BoLA complex and traits of innate and adaptive immunity, and may also help to unravel the mechanisms of resistance to parasitic infections.

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